GROWTH AND TITRATION OF NEWCASTLE DISEASE AND INFECTIOUS BRONCHITIS VIRUSES IN TISSUE CULTURE

by

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INTRODUCTION

Almost from the time that tissues were first grown in vitro, the techniques employed have been exploited by the virologist. It was not until recently, however, that virus investigators have shown anything but interest in composing impressive lists of viruses that could be propogated in vitro. Relatively few attempts were made to explore the potentialities of tissue culture as a practical and convenient means of assaying viral infectivity, of measuring neutralizing antibodies, of altering viral pathogenicity, of producing large quantities of virus suitable for the study of its physical and chemical properties, or for use as an immunizing agent. This failure of the virologist to investigate more vigorously such applications of tissue culture methods may be attributed chiefly to the fact that no simple quantitative means was available to determine within the culture itself whether virus multiplication had occurred.

The first systematic use of tissue culture to detect and titrate virus was Huang's (1942) investigation of the effects of Western equine encephalomyelitis on chick embryo cells. Since then the use of tissue culture in virology has steadily increased until the present time, and this progress was climaxed recently by the monumental work of Nobel prize winners Enders, et al. (1949), in the cultivation of the three strains of poliomyelitis viruses in vitro in non-neural tissue. This, in turn, provided the means for the production of the Salk vaccine.

With the advent of tissue culture methods for studies of poliomyelitis viruses, many techniques for measuring virus activity and neutralizing antibody were developed. One of the new techniques

is a tissue culture test developed by Salk, et al. (1954) using the color of phenol red as the indicator for poliomyelitis virus and antibody activity. This method of detection of viral activity affords a rapid, simple and accurate means for the titration of animal viruses.

The purpose of this investigation was twofold; To demonstrate the multiplication of Newcastle and infectious bronchitis viruses in tissue culture, and to determine whether the color differentiation tissue culture techniques can be employed in titration and neutralization tests of such avian viruses as Newcastle and infectious bronchitis.

REVIEW OF LITERATURE

The intrinsic value of tissue culture as applied to the problem of virus multiplication was clearly demonstrated by Carrel (1924). Employing pure cultures of chicken macrophages, he was able to show that these cells were capable of supporting multiplication of Rous sarcoma virus. It was also demonstrated that an infected cell often assumed one of the characteristics of malignancy; i.e., increased ability to digest the medium. His observations also suggested that multiplication of this virus within the cell did not cause destruction of the cell and that the cell would grow and liberate viruses over an extended period of time.

Four important concepts were established by these experiments of Carrel's: (1) cells in tissue culture infected with a virus may show alterations that can be directly observed in the culture; (2) large quantities of virus may be produced in the system and this multiplication of the virus may continue over long periods of time; (3)

certain viruses may establish a symbiotic relationship with the cell; (4) one type of cell may support growth, whereas another type of cell from the same host may be resistant.

The work on Rous sarcoma virus in plasma clot cultures led Carrel and Rivers (1927) to study vaccinia virus under similar conditions, and it was found that this agent behaved in a like manner. Rivers and associates (1935, Li and Rivers) used the suspended-cell culture method which was developed by Rous and Jones (1915) for the production of vaccinia virus in sufficient quantities for the vaccination of man. Unfortunately, after repeated passage in vitro, the immunizing properties of the virus became reduced, and the tissue culture vaccine was not adapted as a routine method of vaccine production.

Theiler and his associates (1937, Hagen and Theiler 1932) were able to clearly demonstrate that tissue culture could be effectively employed in the development of a virus strain of attenuated virulence suitable for the production of a vaccine. Theiler's cultivation of yellow fever virus in tissue culture resulted in the production of the attenuated strain D17 that is today employed in vaccination.

The importance of being able to recognize the presence of viruses in tissue culture by direct observation was appreciated by the early workers. For example, Steinhardt and his collaborators (1913) tried to demonstrate the production of inclusion bodies by vaccinia virus in vitro. They failed to find Guanieri bodies in their cultures, but they succeeded in demonstrating multiplication of vaccinia virus. Other workers (Harde, 1916, and Haagen, 1929) likewise were not able to demonstrate typical inclusions of vaccinia in tissue culture. It was not until 1929 that unequivocal evidence for the appearance of

such morphologic indicators of viral activity was obtained. Rivers, et al. (1929) and Andrewes (1929) were able to show the presence of inclusion bodies in their tissue culture systems. Andrewes (1929), during the course of these experiments, showed that it was possible to titrate viral neutralizing antibodies in tissue cultures using the suppression of the formation of inclusion bodies as the index of neutralization.

Although the presence of inclusion bodies in tissue cultures may be regarded as evidence for virus multiplication, the development of these structures is irregular and the procedures involved in rendering them visible are time-consuming and laborious. The presence of these structures cannot be taken as conclusive evidence of virus activity since investigators (Andrewes, 1929) have observed similar formation in cells of tissue culture to which no virus had been added. Also, many viruses do not produce inclusion bodies. For these reasons, assay of virus multiplication and of virus neutralization based on inclusion bodies proved of little value for routine work.

Various workers from time to time observed other effects of viruses on cultured cells but their value as means of titration was at first overlooked. Plotz and Ephrussi (1933) reported that cells infected with fowl plague failed to migrate or reproduce normally in plasma clot cultures. Ivanovics and Hyde (1936) noted destruction of various tissues cultured in vitro that were infected with Virus III, but Huang (1942) must be given credit for first recognizing that the destructive capacity of equine encephalomyelitis virus could be utilized for measuring infectivity and for assaying the neutralizing capacity of immune sera. Huang showed that the infectivity titer of

equine encephalomyelitis virus could be assayed with accuracy in vitro by determining the highest dilution which would inhibit chick embryo cells from migrating in plasma cultures. He then, by using the constant serum-varying virus technique, was able to determine the neutralizing index of the serum by measuring prevention of injury to the cell cultures. Comparative experiments with tissue culture and mice revealed close agreement in measurements of infectivity and neutralizing antibodies. Indeed, under certain conditions, tissue culture appeared to be even more sensitive in the measurement of these properties. This tissue culture system revealed a simple and accurate means for assay of those viruses which have the capacity to injure or kill cells when cultured in vitro.

Enders, et al. (1949) propagated poliomyelitis virus in a variety of human embryonic tissues, and these workers (Robbins and Enders, 1950) noted the marked cellular destruction caused by virus infection. Others (Smith, et al. 1950; Younger, et al. 1952; Ledinka and Melnick, 1953) soon found that poliomyelitis virus would also propagate in monkey tissues with comparable cytopathogenic effects.

Gey and Bang (1951) stated that they were able to produce focal necrosis in static slide cultures of a rat sarcoma with the virus of Eastern equine encephalomyelitis. Following this work, Dulbecco (1952) developed a sensitive method of titration depending on this virus effect on infected cells. Dulbecco demonstrated the production of plaques by single virus particles of Western equine encephalomyelitis and Newcastle disease virus in monolayers of chicken embryo fibroblasts grown in vitro. Dulbecco and Vogt (1954) re-introduced the Rous-Jones trypsinization procedure for obtaining cells directly from minced tissue and

were then able to grow them on glass surfaces in the absence of a plasma clot.

There are two general techniques which are used for virus neutralization tests. In one, the concentration of the antiserum is held constant and the virus concentration varied and in the other, the antiserum concentration is varied. The majority of workers who employ the neutralization tests use the constant virus-varying serum technique. The experimental evidence which recommends this procedure has been stated by Tyrrell and Horsfall (1953).

In performing the test using the constant virus-varying serum technique, serial dilutions of the serum were prepared and each dilution was mixed with an equal volume of a virus suspension containing a fixed number of infecting doses of viruses for the tissue culture system employed. After fixed time intervals, equal amounts of each serum-virus mixture was inoculated into two or three cultures, and the cultures were examined for cell destruction after specified time intervals. The neutralizing titer was defined as the highest dilution of serum which gave complete protection against the cytopathogenic effect of the virus.

The investigators found that the quantity of viral antigen was one of the variables that influence the results. Enough virus was needed to produce rapid cellular destruction; insufficient virus produced spottiness of results; larger doses concealed antibody effects.

Ledinko, et al. (1952) studied the quantitative relationship between the amount of Y-SK virus employed and the titer of specific immune sera in monkey testicular tissue cultures. These workers

reported a direct logarithmic ratio between the serum titer and the quantity of virus used.

Under the experimental conditions developed by Salk, et al. (1954), the production of a yellow color by cell cultures in a medium containing phenol red is regularly prevented by un-neutralized poliomyelitis virus. Thus, a simplified method was made available for the titration of the poliomyelitis viruses and their type-specific antibodies. The observation of inhibition of acid formation in infected cultures had been noted in earlier studies with Western equine encephalomyelitis virus and Newcastle virus by Huang (1943) and with poliomyelitis viruses by Enders, et al. (1949).

Salk, et al. (1954) reported that the antibody titers with the color test were approximately three times higher than those found with the cytopathogenic test using poliomyelitis virus in various roller-tube systems. It has been pointed out that in the color test, traces of antibody would be noted by their ability to delay virus activity. In other systems, any degree of severity of cultural degeneration was considered a failure of the serum's ability to neutralize the virus.

The sealing of tubes in the color test by the insertion of a rubber stopper into each tube has been, in some cases, replaced by a heavy mineral oil which was layered directly on the culture fluid to achieve the desired exclusion of air. When heavy mineral oil was used, the slight change due to cellular metabolism was offset by reversion to red due to leakage of the CO_2 through the oil film (Melnick and Opton, 1955).

Rightsel et al. (1956) described a simple, rapid and inexpensive technique for performance of virus assays in tissue culture systems

employing a disposable multipurpose plastic container. This method is an extension of the color test and is in use in many virus diagnostic laboratories.

The multiplication of fowl plague in cultures of chicken embryo tissues was described by Marchoux (1908). Since then, there have been reports on the growth of this virus and a related virus, Newcastle disease (Hallawer, 1931; Tapacio, 1934; Plotz, 1937; Bankowski and Boynton, 1948; Flewitt and Challice, 1951; Gey and Bang, 1951; Dulbecco, 1952). Essentially all of the above reports were preoccupied with the multiplication of these viruses in cultures of chick embryo tissues.

The multiplication of fowl plague and Newcastle disease viruses in roller-tube tissue cultures employing chick fibroblasts and epithelial tissues was accomplished by Perreira and Gampels, (1954). They followed multiplication by the observation of hemagglutinin cytopathogenic effects and the pH differential test.

Cytopathogenic changes which occur in tissue cultures infected with the viruses of Western equine encephalomyelitis and Newcastle disease viruses were described by Fastier (1954). He then used this change as a means of titration of specific immune serum.

Bang and Warwick (1954) tried unsuccessfully to produce an avirulent strain of Newcastle disease virus in tissue culture. Indeed, they noted an apparent increase in virulence for chickens after repeated tissue culture passage.

The propagation of influenza and Newcastle disease virus in HeLa cell cultures was demonstrated by Tyrrell (1955); that both viruses caused cytopathogenic changes was also noted. Mascoli (1956)

studied extensively the action of incomplete, virulent and two vaccine strains of Newcastle disease virus on chick embryo fibroblasts cultured in vitro and found them essentially identical in their effects.

Rubin and Franklin (1957) have shown Newcastle disease virus to be neutralized by immune serum in a manner which implies that only one antibody molecule is necessary for inactivation of a single virus. Plaque formation on monolayer tissue cultures of sixteen day old chicken embryo lung tissues was employed as the titration method.

Fahey and Crawley (1956) reported on the propagation of infectious bronchitis virus in tissue culture. They found that chorioallantoic membrane tissue fragments would support infectious bronchitis virus multiplication for long periods without apparent damage to the cells. They also reported that cultures of whole chick embryo, chick embryo heart and monkey kidney cells were infected by infectious bronchitis without damage to the cells. Growth of the virus was followed by titration in embryonated chicken eggs.

The preceeding literature review reveals that a sensitive titration procedure has been developed for titration of poliomyelitis virus and antiserum. It was also noted that the multiplication of both Newcastle disease and infectious bronchitis in tissue culture has been demonstrated.

MATERIALS AND METHODS

Viruses

Infectious bronchitis virus (IBV strain V114D) was obtained from Dr. Cunningham, Michigan State College and Newcastle disease virus

(NDV), California strain (1194) and L.W. strain from Kansas State College.

Stock cultures of both IBV and NDV were prepared by passage in 9 or 10 day old chick embryos. After 72 hours, or earlier death of the embryos, the allantoic fluids were harvested. Two ml amounts were sealed in glass vials, quick frozen and stored in a dry ice box. EI_{50} and HA titers were determined, and periodic checks were made to assure high titer stocks.

Medium

Hank's and Wallace (1949) or Gey's (1936) balanced salt solutions (BSS) was used for making dilutions and for preparing growth medium when it was desired in the tissue culture systems. Medium 199 described by Morgan, et al. (1950) was used as a maintenance medium and was purchased from Difco in 100 ml self-sealing vials.

Horse serum was purchased from Microbiological Associates, Inc. in 25 ml volumes. This serum was found to be more uniform and of more reliable quality for tissue culture purposes than that available at Kansas State College. The horse serum was frozen and kept at -25° C until used. The frozen horse serum was thawed and heated at 56° C for 30 minutes just before use. This was done to redissolve substances which had precipitated upon freezing.

Human serum was purchased from Microbiological Associates, Inc. or obtained through the courtesy of Mr. E. Mora of Kansas State College. The pooled human serum was frozen and kept at -25° C until used. Before use the serum was filtered through a #03 Selas filter and heated at 56° C for 30 minutes.

Prior to use, penicillin and streptomycin (100 units of each antibiotic per ml) were added to all tissue culture media.

Embryo extract was prepared in the following manner. Nine or ten day old embryos were removed from their eggs and the whole embryos placed in a Waring blender. An equal volume of Hank's or Gey's BSS without $NaHCO_3$ was added and the embryos broken up. Care was taken to reduce foaming to a minimum. The resultant mixture was then placed in a water bath at 56° C for 30 minutes. The pulp was filtered through two layers of cheese cloth and centrifuged at 20,000 x G with an angle head centrifuge for 30 minutes. The extract was sterilized and clarified by filtration through a Selas #03 filter employing the following method. A partial vacuum was placed on the system by use of a water aspirator for one hour and the vacuum maintained by clamping the hose between the aspirator and flask. The flask and filter were then placed in the refrigerator at 4 C (which greatly increased the vacuum). After filtration had stopped, the clear fluid was removed and 5 ml amounts were placed in 20 ml dilution bottles. The embryo extract was stored at -25° C and sterility was checked by inoculating thioglycollate medium and looking for bacterial growth over a period of seven days.

Embryo Infectivity Titrations

In all titrations 9 to 11 day old embryos were employed. A minimum of five eggs per dilution was used. Ten fold serial dilutions of the virus to be titrated were prepared in physiological saline with 500 units of both penicillin and streptomycin added per ml. One-tenth ml of the virus dilution was inoculated into the allantoic cavity of

each egg with a 1 ml tuberculin syringe, using a ½ inch, 26 gage needle. Incubation of the inoculated eggs was at 37° C. Embryos dying during the first 24 hours, were discarded from the titration calculations. The titrations were teminated after five days, and EI_{50} end points were calculated by the method of Reed and Muench (1938).

Hemagglutination Titers

The hemagglutination titers were performed by diluting the virus two-fold serially in 0.25 ml of physiological saline and adding 0.25 ml of 0.5 per cent chicken red blood cell suspension in physiological saline. This is a modification of the method of Doll, et al. (1950).

Hemagglutination-Inhibition (HI) Titers

The method of Beach (1948) was modified slightly and used for all HI titrations. Two-fold dilutions of serum on 0.25 ml saline ranging up to 1:2560 or higher were prepared. Antigen, 10 HA units in 0.25 ml amounts was added to each dilution of serum. The serum-virus mixture was incubated at room temperature for 10 minutes and 0.25 ml of a 0.5 per cent RBC suspension added. The series was read when the RBC control tube showed a definite button pattern. This was usually around 30 minutes.

Tissue Culture Procedures

Trypsinization of chicken embryo tissues was performed at either 37° C for 15 minutes (Rous and Jones, 1915) or at 4° C for approximately 18 hours (Bodian, 1956). The procedure for cold trypsinization differed from trypsinization at 37° C only in time and temperature. Ten, 9 or

10 day old embryos were decapitated and washed twice with 30 ml of BSS. The embryos were then expressed through a 10 ml syringe into 10 ml of 0.5 per cent trypsin solution in BSS. The resulting tissue suspension was then placed in the refrigerator at 4° C for approximately 18 hours. Then an equal volume of BSS was added and the suspension filtered through two layers of cheese cloth and centrifuged at 500 RPM with a Clay-Adams Safeguard horizontal centrifuge for 20 minutes. The cells were then washed once with 10 ml of BSS, centrifuged and susponded in 20 ml of BSS for counting with a hemocytometer.

This method of trypsinization gave a high yield of viable cells even after 48 hours of trypsinization. Cell aggregation appeared to be less frequent when cold trypsinization was employed.

Minced allantoic or amnionic membranes were prepared by removing the membranes from 12 or 13 day old embryonated chicken eggs. The whole membranes were then minced with two Bard-Parker knives to yield small membrane fragments about 3 mm square. The minced membranes were then suspended in 10 ml of BSS in bottles which were incubated at 35° C.

HeLa cell stock cultures were maintained using a medium consisting of 80 per cent Difco 199, 10 per cent human serum (HuS) and 10 per cent horse serum (HS). Trypsinization of the stock cultures was carried out at 37° C for 30 minutes using a 0.5 per cent trypsin solution. The suspended cells were washed twice with BSS and then counted in a hemocytometer.

Titration Procedure

Replicate cultures were prepared using either 13 x 100 mm test tubes or 13 x 50 mm flat-bottom screw cap vials. The use of flat-bottom

vials was preferred as the cells were able to spread evenly over the flat surface. White rubber #00 stoppers purchased from the West Rubber Co. were used to seal the tubes or vials.

The cells were suspended in the medium used for the test at a known concentration, and a magnetic stirrer was used to keep the cells suspended uniformly throughout the medium. An automatic Cornwall pipette was used to deliver a constant volume of the cell suspension to each of the tubes or vials to be used in the test. The virus or virus-antiserum mixture was added, and then the tubes or vials stoppered. Incubation was carried out with the cultures in an upright position and the temperature maintained at the desired level, usually at 35° C.

EXPERIMENTAL RESULTS

Multiplication of Newcastle Disease Virus in Tissue Culture

Growth of NDV in Chicken Embryo Fibroblasts. Medium A contained 45 per cent Hank's BSS, 45 per cent serum and 10 per cent embryo extract. Medium B contained 90 per cent Difco 199 and 10 per cent serum. A cell suspension employing medium A was prepared to give 3×10^6 of freshly trypsinized cells per ml. Three screw cap tubes were inoculated with one ml each of the cell suspension and one bottle was inoculated with 10 ml of the cell suspension. After 24 hours, the cell sheet was washed once and 1 ml of medium B added to each of the tubes and 10 ml of medium B added to the bottle culture. Stock NDV, with an EI_{50} titer of 10^{-9} and a HA titer of 2560, was then diluted in Hank's BSS to 10^{-5} and 0.1 ml of the 10^{-5} dilution added to two tube

cultures and the bottle culture.

The EI_{50} titers of the fluid in the bottle culture are listed in Table 1 showing rapid growth of NDV in this tissue culture system.

Table 1. NDV multiplication in chick embryo fibroblasts.

Time :	0 hours :	12 hours :	24 hours
EI_{50} titers	$10^{-4.48}$	$10^{-4.66}$	$10^{-8.5}$

Comparison of the two infected tubes and the one noninfected tube with the low power objective of the microscope showed no dectectable difference. When an attempt was made to stain with neutral red dissolved in Difco 199, sections of cells in the tubes inoculated with the virus did not adhere to the glass surface. The uninoculated tube did not show this effect, indicating that infected cells lose their ability to adhere to the glass surface.

NDV Multiplication in HeLa Cells. The medium contained 75 per cent Difco 199, 10 per cent horse serum and 15 per cent human serum. Leighton culture tubes containing a continuous monolayer of HeLa cells were washed once with complete medium and 2 ml of complete medium then added to each tube. NDV stock (EI_{50}, $10^{-9.5}$; HA, 2560) was diluted 10^{-3} and 0.05 ml added to each of 6 culture tubes. Three culture tubes received 0.05 ml of normal allantoic fluid and were used as controls. All the tubes were incubated at 35° C at a slant so as to cover the cell sheet with medium.

Table 2. Cytopathogenic effects of NDV cultured in HeLa cells.

	Time		
	0 hours	24 hours	72 hours
Cytopathogenic effects in infected tubes.			
1	neg.	1+	*4+
2	neg.	neg.	4+
3	neg.	2+	4+
4	neg.	neg.	4+
5	neg.	neg.	4+
6	neg.	1+	4+
Controls			
1	neg.	neg.	neg.
2	neg.	neg.	neg.
3	neg.	neg.	neg.

*4+ = maximum cell destruction

After 72 hours definite cytopathogenic effects could be observed in the infected cultures. This was compared to the normal cell sheets in the control tubes. Table 2 contains the results of this experiment and the results show definite NDV multiplication in HeLa cells as determined by cytopathogenic effects.

NDV multiplication in HeLa cells was also shown by following the production of infectious virus. The medium used consisted of 5 per cent serum and 95 per cent Difco 199. Four bottle cultures of HeLa cells were washed with the above medium once, and then 10 ml of medium were added. Two of the bottle cultures received 0.1 ml of a 10^{-3} dilution of NDV stock with an EI_{50} titer of $10^{-8.9}$ and a HA titer of

1280. The two remaining bottle cultures were kept as controls on the cells.

At 24 hour intervals 0.2 ml samples were removed, quick frozen and stored for later titration. The experimental results, listed in table 3, show NDV multiplication with a maximum titer being reached at approximately 48 hours.

Table 3. NDV multiplication in HeLa cells.

	Time			
	0 hours	24 hours	48 hours	72 hours
EI_{50} (a)	$10^{-3.1}$	$10^{-4.7}$	$10^{-7.2}$	$10^{-7.1}$
EI_{50} (b)	$10^{-4.0}$	$10^{-5.2}$	$10^{-6.8}$	$10^{-7.4}$
Cytopathogenic effects				
(a)	neg.	neg.	3+	4+
(b)	neg.	neg.	2+	4+
Controls				
Cytopathogenic effects	neg.	neg.	neg.	neg.

Multiplication of Infectious Bronchitis Virus in Tissue Culture

Storage of Stock Cultures of IBV. An experiment was performed to determine the best method of storage of IBV. One ml of allantoic fluid containing IBV with a titer of $10^{-7.5}$, as determined by EI_{50}, was placed into each of 24 two ml glass vials. The vials were then sealed with a flame. Eighteen of the vials were then quick frozen in a dry ice-alcohol mixture.

Storage at three different temperatures was accomplished by using (1) a deep freezer at -20° to -25° C, (2) the freezer compartment of a refrigerator at -10° to -15° C, and (3) a dry ice cabinet at approximately -60° to -65° C. The results are shown in Table 4, and demonstrate clearly the value of quick freezing and storage at low temperatures. It may be seen that storage at -60° C is best. In accordance with this, all stocks of viruses were quick frozen and stored in the dry ice cabinet to maintain a relatively stable titer.

Table 4. Viability of IBV upon storage at various temperatures.

	Temperature of storage		
	-10° C	-20° C	-60° C
Quick frozen:			
EI_{50} titer			
15 days	$<10^{-1}$	$10^{-1.5}$	$10^{-7.2}$
30 days	$<10^{-1}$	$<10^{-1}$	$10^{-7.4}$
52 days	neg.	neg.	$10^{-6.9}$
Not Quick Frozen:			
EI_{50}			
15 days	$<10^{-1}$	$<10^{-1}$	$10^{-5.1}$

Multiplication of IBV in Chicken Embryo Fibroblasts. The media used consisted of 50 per cent Difco 199, 30 per cent serum and 20 per cent embryo extract. One and nine-tenths ml of a cell suspension containing 2×10^6 cells per ml was added to each of two screw cap tubes. To each of these tubes was added 0.1 ml of IBV stock with an EI_{50}

titer of $10^{-7.2}$. At specified time intervals, 0.2 ml samples were removed from each tube and EI_{50} titers determined. The results are listed in Table 5. Although there is a slight rise in titer after thirty minutes, the extreme drop noted at 24 hours indicates that the system does not support IBV multiplication to any extent.

Table 5. Propagation of IBV in chicken embryo fibroblasts.

	Time		
	0 minutes	30 minutes	24 hours
EI_{50} titer tube (a)	$10^{-4.5}$	$10^{-5.1}$	$< 10^{-1}$
EI_{50} titer tube (b)	$10^{-4.8}$	$10^{-5.4}$	$10^{-1.1}$

Growth of IBV in the Cells of the Chorioallantoic Membranes (CAM) of Chicken Embryonated Eggs. An attempt to disperse the cells of the CAM of 9-day-old embryos with trypsin met with failure. This was probably caused by the extensive length of time required for exposure of the cells of the CAM to the action of the trypsin before dissolution of the cementing material of the membranes.

The medium used consisted of 60 per cent Difco 199 and 40 per cent serum. Ten small fragments of CAM from two, 9-day-old embryonated eggs were placed into each of two, 13 x 100 mm tubes and 0.9 ml of medium added. One-tenth ml of IBV stock (titer $10^{-6.2}$) was added to each tube and the tubes then stoppered and incubated at 37° C. At 0 minutes, 30 minutes and 24 hours, 0.2 ml samples were removed, quick frozen and stored at -60° C for titration at a later date. The results are listed in Table 6.

The cells caused a rapid drop in the pH of the medium showing their viability and the lack of destruction due to viral action. The drop in IBV titer indicates the failure of this tissue culture system to support the growth of IBV.

Table 6. IBV in cells of the CAM.

	Time		
	0 minutes :	20 minutes :	24 hours
EI_{50} titer tube (a)	$10^{4.2}$	10^{3}	$<10^{-1}$
EI_{50} titer tube (b)	$10^{4.0}$	$10^{3.8}$	$<10^{-1}$

IBV in Cells of the Amnionic Membranes (AM) of Chicken Embryonated Eggs. This experiment was performed in a similar manner to that using CAM. The titer of the stock virus was $10^{-6.8}$. From the results, which are listed in Table 7, there is again noted failure of these cells to support growth of IBV.

Table 7. IBV in cells of the AM.

	Time		
	0 minutes :	20 minutes :	24 hours
EI_{50} titer tube (a)	10^{-3}	$10^{-3.4}$	$<10^{-1.1}$
EI_{50} titer tube (b)	$10^{-3.3}$	$10^{-3.0}$	$<10^{-1.0}$

IBV in HeLa Cells. The medium used in this experiment contained 90 per cent Difco 199 and 10 per cent serum. The old medium was poured from three 20 ml dilution bottles which contained a continuous layer of HeLa cells attached to the glass surface. The cell sheets were washed once and 3 ml of the above medium added. Twenty-five hundredths of a ml of IBV stock with an EI_{50} titer of $10^{-5.9}$ was added to two of the bottle cultures thus prepared. Results listed in Table 8 show that HeLa cells did not support IBV multiplication.

Table 8. IBV in HeLa cells.

	Time		
	0 minutes	30 minutes	24 hours
EI_{50} titer tube (a)	$10^{-4.1}$	$10^{-1.9}$	$<10^{-1}$
EI_{50} titer tube (b)	$10^{-3.8}$	10^{-2}	$<10^{-1}$

The results of these attempts to show IBV multiplication in tissue culture are in disagreement with work published by Fahey and Crawley (1956). Fahey was able to show multiplication of IBV in CAM, fibroblasts and heart cells of the chick embryo and monkey kidney cells cultured in vitro. The discrepancy may be that Fahey used serial passage with 48 hours between transfers. There was also a difference in virus strains used.

Titration of NDV and IBV by Tissue Culture Methods

Effect of Normal Allantoic Fluids on the Tissue Culture Systems. This experiment was performed to demonstrate that normal allantoic

fluid (NAF) did not cause any change due to pH of the allantoic fluid or by changing the metabolism of the cells.

The medium consisted of 70 per cent Difco 199, 20 per cent serum and 10 per cent embryo extract. One ml of the medium, containing 1×10^6 chick embryo fibroblasts per ml, was added to each of 33 tubes. Then NAF, which had been frozen for 14 days, was thawed and centrifuged in the same manner as infected allantoic fluids used in the titration procedure. The NAF was diluted ten fold, serially 10^{-1} to 10^{-6} in BSS and 0.1 ml from each dilution inoculated into each of five tubes per dilution. The three control tubes received 0.1 ml of BSS.

After 24, 48 and 72 hours the cells were metabolizing and there was a uniform color change in all tubes. The experiment was terminated after 72 hours because the pH had fallen below the indicator level of phenol red. A dilute solution of methylene blue was added to all tubes forming a greenish-blue color. The color was of uniform density throughout the titration series. The uniform color change before and after addition of the methylene blue indicates that NAF did not effect the titration procedure.

Colormetric Titration of NDV Stock Using Replicate Tissue Culture Vials of Chicken Embryo Fibroblasts. The medium used consisted of 90 per cent Hank's BSS and 10 per cent serum.

Cells were prepared by trypsinization at 37° C. To the above medium were added cells to give 1×10^6 cells per ml. Five vials per dilution were prepared, each containing 1 ml of cell suspension. NDV (EI_{50} $10^{-8.1}$) was diluted ten fold, serially in Hank's BSS and 0.1 ml was added to each vial in the dilution series. The control vials

received 0.1 ml of Hank's BSS. All vials were then capped with white rubber stoppers and placed upright in the incubator at 37° C.

After 48 hours 0.25 ml of a dilute solution of methylene blue was added to all vials. The results of this experiment are shown in Table 9. When this method of titration was repeated results similar to those in Table 9 were obtained, indicating that NDV could be titered by this procedure.

Table 9. Titration of NDV by color end point method in replicate tissue cultures of chick embryo fibroblasts.

	Series				
	1	2	3	4	5
Dilution					
10^{-1}	4+	4+	4+	4+	4+
10^{-2}	4+	4+	4+	4+	4+
10^{-3}	4+	4+	4+	4+	4+
10^{-4}	4+	4+	4+	4+	4+
10^{-5}	3+	4+	3+	2+	4+
10^{-6}	2+	2+	1+	0	0
10^{-7}	0	0	0	0	0
10^{-8}	0	*1+	0	0	0
10^{-9}	0	0	0	*4+	0

Scoring is based on comparison with color change in control tubes.

*Degeneration of culture due to causes other than inoculated virus.

An experiment using the same method as the above was performed with

HeLa cells as the susceptible cells. The results obtained were similar to those shown in Table 9 with a slightly lower positive response.

The Ability of Methylene Blue to Act as the Sole Indicator in the Titration Procedure Using Color Change as the End Point. The titration was set up similar to all the other runs, using chick embryo fibroblasts infected with NDV, except that Hank's BSS without phenol red was used as the diluting salt solution.

After 72 hours a dilute solution of methylene blue was added to each vial, and the series was observed for any color difference corresponding to the virus dilutions. There was a very slight detectable difference using just methylene blue, but when phenol red indicator in Difco 199 was added, the difference was magnified and a titer of 10^{-5} plus was recorded.

Titration of the Neutralizing Capacity of Immune Sera, Specific for NDV, in Tissue Culture

The medium used in this experiment consisted of 90 per cent Hank's BSS and 10 per cent serum. The immune sera were prepared by series inoculation of rabbits with L. W. and California strains of NDV. The hemagglutination inhibition titers of the sera were 1600 and 800, respectively.

NDV stock was titered in tissue culture using color change as the end point. The dilution of virus, 10^{-5}, was found to give consistent positive results in the tissue culture system employed. The neutralization test was set up as follows.

Freshly cold-trypsinized fibroblasts were suspended in medium to give a concentration of 1×10^{6} cells per ml. One ml of this cell

suspension was pipetted, with a 2 ml Cornwall automatic pipette, into each of 60 vials. The antiserum prepared against NDV strain L. W. was diluted two fold serially starting with a 1/5 dilution to 1/2560. One-tenth ml of the serum dilutions was added to five vials for each dilution.

The NDV stock was diluted to 10^{-4} in the same type medium as the cells and serum. One-tenth ml samples of the virus dilution were added to each of 40 of the vials containing immune serum and cells and to 10 vials containing just cells. The remaining 10 vials containing just immune serum and cells received 0.1 ml of the diluting medium to bring the volume to 1.1 ml. The vials were then sealed with stoppers and incubated at 34° C. After five days, the neutralization titer of the serum was determined by comparison of the color change in the serum and virus control vials. The results of the titration are listed in Table 10. The 1/5 serum was observed to be toxic to the cells, and these tubes were discarded.

Table 10. Neutralization test of NDV immune sera in tissue culture.

Serum Dilution	Virus Added (Replica tests) 1	2	3	4	Serum Control	Virus Control No immune Serum
5	discarded				discarded	4+
10	neg	neg	neg	neg	neg	4+
20	neg	neg	neg	neg	neg	4+
40	neg	neg	neg	neg	neg	4+
80	neg	1+	1+	neg	neg	4+
160	2+	3+	2+	neg	neg	4+
320	4+	4+	4+	4+	neg	4+
640	4+	4+	4+	4+	neg	4+
1280	4+	4+	4+	4+	neg	4+
2560	4+	4+	4+	4+	neg	4+

Definite protection against virus attack at dilutions of 1-40 with partial protection of dilutions 1-80 and 1-160 was noted. When this was repeated definite protection was found to be again at a dilution of 1-40 with partial protection at 1-80 and 1-160.

The above neutralization experiment was repeated using immune sera prepared against the virus stock, California strain 1194. In three separate runs, complete protection was noted in all titration series at a dilution of 1-20 with partial protection at 1-40 and 1-80.

IBV Titration in Tissue Culture. This test was performed to determine if the cessation of acid formation by the cells due to virus attack would give a method by which pH determination could be used for an end point in titration.

The medium used contained 90 per cent Difco 199 and 10 per cent serum and was buffered to pH 7.7 with $NaHCO_3$.

Decimal dilutions of the virus stock were prepared in media without cells, 10^{-1} to 10^{-9} inclusive. To 13 x 100 mm tubes, 0.9 ml of medium containing 1×10^6 chicken embryonic tissue cells were added. To each of six tubes containing cells, was added 0.1 ml of the first dilution, which was repeated for each dilution. The tubes were tightly stoppered and placed upright in the incubator at 35° C.

As can be seen from Table 11, the method did not afford a good means of titration of IBV. There was noted a slight difference of pH corresponding to virus dilution but there was no significant difference.

Table 11. pH of the fluid phase of IBV infected serial tissue culture tubes.

		Time			
		12 hours	24 hours	48 hours	72 hours
Dilution					
10^{-1}	pH	7.68	7.62	7.3	7.2
10^{-2}		7.7	7.59	7.35	7.0
10^{-3}		7.72	7.56	7.2	7.1
10^{-4}		7.68	7.58	7.25	6.2
10^{-5}		7.64	7.53	7.2	7.2
10^{-6}		7.66	7.51	7.3	6.9
10^{-7}		7.65	7.52	7.1	6.5
10^{-8}		7.6	7.5	7.25	7.0
10^{-9}		7.63	7.54	7.29	7.2
Controls		7.64	7.51	7.2	7.0

Other runs were performed varying the cell concentration, initial pH, time interval and nutrient medium. All such runs gave results that were similar to the above.

Color Difference between IBV Infected and Non-infected Tissue Culture Tubes Using a Spectrophotometer. The medium used in this experiment consisted of 70 per cent Gey's BSS, 20 per cent serum and 10 per cent embryo extract. One hundred, 13 x 100 mm, tubes were standardized as to uniform light deflection in a spectrophotometer. Nine-tenths ml of a cell suspension, containing 1.2×10^6 cells per ml, was added to each of 80 standardized tubes.

A sample of IBV with a titer of $10^{-6.2}$ was diluted ten fold serially, 10^{-1} to 10^{-10} dilutions inclusive. One-tenth ml was inoculated into eight tubes of each dilution, and all tubes were tightly sealed with rubber stoppers.

At set time intervals, two tubes were removed from each dilution and dilute methylene blue was added so that a good color density was obtained. The tubes were again stoppered and readings were taken with the spectrophotometer using a wave length of 600 mu; the results were then averaged, as listed in Table 12. There was noted slight differences corresponding to dilution at 14 and 22 hours but this difference was not enough to be used for titration purposes.

Table 12. Spectrophotometer readings of replicate tissue culture tubes infected with dilutions of IBV.

	Time			
	14 hours	22 hours	48 hours	72 hours
Dilution				
10^{-1}	43.5	28.0	27.5	30.0
10^{-2}	37.0	26.5	27.2	31.5
10^{-3}	36.5	28.5	27.7	29.5
10^{-4}	40.0	26.5	27.0	31.0
10^{-5}	35.0	26.5	28.0	28.5
10^{-6}	37.0	26.0	29.0	31.7
10^{-7}	37.5	26.0	26.8	29.2
10^{-8}	30.5	25.5	25.0	30.2
10^{-9}	32.8	24.5	28.0	31.2
10^{-10}	32.5	24.0	27.5	20.9

Wave length: 600 mu

An experiment was performed with the culture tubes prepared in a manner similar to the above, in which all tubes were inoculated with the indicator at 16 hours and read at that time. The results are listed in Table 13.

A sharp drop was noted in the light transmittance at dilution 10^{-8} in series 1 and 5 and a similar sharp drop in dilution 10^{-10} in series 2, 3 and 4. This experiment was repeated and results essentially similar to those in Table 13 were noted. There was a definite difference in light transmittance that tended to correspond to virus dilution but the results were erratic, and definite titer could not be assigned to the virus samples.

Table 13. Spectrophotometer readings of replicate tissue culture tubes infected with IBV. Readings were taken at 16 hours.

	Series				
	1	2	3	4	5
Dilution					
10^{-1}	20	16	21.5	13.5	17.6
10^{-2}	19	12	16.5	13	16.5
10^{-3}	15	12.2	17.5	13.7	17.5
10^{-4}	18	13.3	18	15	17
10^{-5}	18	12.5	16.5	14	18
10^{-6}	14.5	12.7	17	14	16.2
10^{-7}	16.2	13.5	16	16	17.5
10^{-8}	10	10	17.5	14	9.2
10^{-9}	10.2	13	17.5	20.5	12
10^{-10}	10.3	11	10.5	7	11

(wave length: 600 mu)

DISCUSSION

The ability of NDV to rapidly multiply in various tissue culture systems causing extensive cell destruction has been pointed out by many investigators. The replicate tissue culture titration system developed in this laboratory depended upon rapid cell destruction. The NDV infected cells in the culture tubes were destroyed, and the pH of the infected tubes was not lowered to the extent of the non-infected tubes. The pH difference was noted by the color of phenol red and methylene blue, the methylene blue being added at the end of the incubation period.

The sensitivity of the replicate tissue culture titration technique was found to be less than when NDV was titered in eggs. It would be possible to increase the sensitivity of this titration method by using mineral oil to seal the tubes in place of stoppers as reported by Younger (1955).

Even though the sensitivity of this tissue culture titration procedure was less than the sensitivity of EI_{50} titration, there are three advantages in using tissue culture in titration, namely: (1) the system can be freed of all antibodies by repeated washing of the cells used, (2) tissue culture is a more uniform system and (3) the cost of tissue culture titration is much less than embryo infectivity titration.

The titration procedure using color change in replicate tissue cultures was found sensitive enough to be used for neutralization tests. The neutralization test developed employed the constant virus-varying serum procedure and proved to be a dependable method for titration of the protective capacity of immune serum. Although the hemagglutination

inhibition test is available for work with NDV immune sera, neutralization tests performed in tissue culture may be of value. Any titration procedure using infectivity would be from 10^5 to 10^6 (Luria, 1953) times more sensitive than would a method using the HI test. The HI test is a measure of the serum's ability to inhibit agglutination of red blood cells, whereas the tissue culture neutralization test is a measure of the serum's ability to inhibit infection of susceptible cells.

In preliminary work the action of methylene blue was found to enhance the color difference caused by the effect of hydrogen ion concentration on phenol red. Methylene blue did not, by itself, give a good color difference; but, when phenol red was present, the difference was accentuated. It appears that a combination of the colors of the two indicators increases color difference at various hydrogen ion concentrations.

Although multiplication of IBV in tissue culture could not be shown in this laboratory by egg inoculation after a single passage in tissue culture for 24 hours, evidence is presented indicating that IBV affect cell metabolism. Fahey and Crawley (1956) reported IBV multiplication without subsequent cell destruction. He also reported that the pH of the culture fluids were not affected. The results shown in Tables 12 and 13 demonstrate that, using a replicate tissue culture system, differences in metabolism of infected and non-infected cells could be detected by differences in the pH of the tissue culture fluids.

The detectable difference between infected and non-infected tissue cultures was not pronounced enough to allow the replicate tissue culture procedure to be used as an accurate titration technique for

IBV. It is possible that, with the proper adjustment of cell concentration and medium employed, the sensitivity of this technique could be magnified to a degree where a tissue culture system could be used as a means of titration.

It should be possible to extend this method of titration to include those avian viruses which do not agglutinate red blood cells but do cause rapid cell destruction in tissue culture. Even those viruses which do not cause cell destruction but do cause some metabolic upset of infected cells may be titered by this method.

SUMMARY

The rapid multiplication of NDV in HeLa cells and chicken embryo fibroblasts was demonstrated. IBV multiplication could not be demonstrated under the testing systems employed, in HeLa cells or in embryo fibroblasts, CAM, and AM.

A replicate tissue culture system for virus titration was developed. This titration method depends upon color change of pH indicators due to the different degrees of metabolism of infected and non-infected tissue cells. Using this method, it was possible to titrate NDV and NDV antiserum with readily reproducible results.

The tissue culture titration system developed could not be used for IBV titration due to lack of sensitivity. However, it was demonstrated that IBV caused some metabolic upset of infected cells in tissue cultures.

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LITERATURE CITED

Andrewes, C. H.
Virus III in tissue culture. I. The appearance of intranuclear inclusions in vitro. Brit. J. Exptl. Pathol. 10:188-190. 1929.

_____.
Virus III in tissue culture. II. Further observations on the formation of inclusion bodies. Brit. J. Exptl. Pathol. 10:273-280. 1929.

Bang, F. B., and A. Warwick
A virulent and an avirulent strain of NDV in tissue culture. Federation Proc. 13:421. 1954.

Bankowski, R. A., and W. H. Boynton
Studies on in vitro cultivation of NDV. Vet. Med. 43:305. 1948.

Beach, J. R.
The application of the hemagglutination-inhibition test in the diagnosis of avian pneumoencephalitis (Newcastle disease). Am. Vet. M. Assn. 1948.

Bodian, David
Simplified method of dispersion of monkey kidney cells with trypsin. Virology. 2:575-576. 1956.

Carrel, A.
Action de L'extrait filtre du sarcome de Rous sur les macrophages du sang. Soc. Biol. Compt. Rend. 91:1069-1071. 1924.

Carrel, A., and T. M. Rivers
La fabrication du vaccin in vitro. Soc. Biol. Compt. Rend. 96:848-850. 1927.

Cox, H. R.
Tissue culture as a more sensitive method than animal inoculation for detecting equine encephalomyelitis virus. Proc. Soc. Exptl. Biol. Med. 33:607-609. 1936.

Doll, E. R., M. E. Wallace and W. H. McCollum
Interpretation of serologic procedures for the diagnosis of Newcastle disease. Am. J. Vet. Res. 11:265-270. 1950.

Dulbecco, Renato
Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Nat. Acad. Sci. 38:747-752. 1952.

Dulbecco, R., and M. Vogt
Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exptl. Med. 99:167-182. 1954.

Enders, J. F., T. H. Weller and F. C. Robbins
Cultivation of the Hansing strain of polio virus in cultures of various human embryonic tissues. Science. 109:85-87. 1949.

Fahey, J. E., and J. F. Crawley
Propagation of infectious bronchitis virus in tissue culture. Can. J. Microbiol. 2:503-510. 1956.

Fastier, L. B.
Infectivity titrations of the viruses of Western equine encephalomyelitis and Newcastle disease by tissue culture methods. J. Immunol. 72:341. 1954.

Flewett, T. H., and C. E. Challice
The intracellular growth of fowl-plaque virus: A phrase contrast and election microscopical study of infected tissue culture. J. Gen. Microbiol. 5:279-287. 1951.

Gey, G. O., and F. B. Bang
Viruses and cells. A study in T.C. applications. Trans. N.Y. Acad. Sci. 14:15.

Gey, G. O., and M. K. Gey
The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer. 27:45-76.

Haagen, E.
Ueber das verhaten des variola-vakzanevirus in der geweberkultur. Zentr. Allgem. Path. Anat. 45:268. 1929.

Haagen, E., and M. Theiler
Studies of yellow fever virus in tissue culture. Soc. Exptl. Biol. Med. Proc. 29:435-436. 1932.

Hallawer, C.
Uber das verhaten von huhnerpestvirus in der gwebekultur. Ztscha. Hyg. 113:61-72. 1931.

Hanks, J. H., and R. E. Wallace
Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med. 71:196-200. 1949.

Harde, E. S.
Some observations on the virus of vaccinia. Inst. Pasteur. Ann. 30:209-306. 1916.

Huang, C. H.
Titration and neutralization of Western strain of equine encephalomyelitis virus in tissue culture. Proc. Soc. Exptl. Biol. Med. 51:396-398. 1942.

_____.
A visible method for titration and neutralization of viruses on the basis of pH changes in tissue culture. Proc. Soc. Exptl. Biol. Med. 54:160-168. 1943.

Ivanovics, C., and R. R. Hyde
A study of rabbit virus III in tissue culture. Am. J. Hyg. 23:55-73. 1936.

Ledinko, N., and J. L. Melnick
Poliomyelitis viruses in tissue culture. V. Reaction of virus and antibody: Variables of quantitative neutralization test. Am. J. Hyg. 58:223-247. 1953.

Ledinko, N., J. T. Riordan and J. L. Melnick
Multiplication of poliomyelitis virus in tissue culture of monkey testes. I. Growth curves of type I (Brunhilde) and type 2 (Lansing) strains and description of a quantitative neutralization test. Am. J. Hyg. 55:323. 1952.

Li, C. P., and T. M. Rivers
Cultivation of vaccine virus. J. Exptl. Med. 52:465-470. 1930.

Luria, S. E.
General Virology. p253. John Wiley and Sons, New York. 1953.

Marchoux, E.
Cited from Bang, F. B. Pathology of virus infected cell. Fed. Proc. 14:617-630. 1908.

Mascoli, C. C.
Properties of virulent, incomplete and vaccine strains of Newcastle disease virus in embryonated eggs and in tissue culture. Ph.D. Thesis, Ohio State University. 1956.

Melnick, J. L., and E. M. Opton
Assay of poliomyelitis neutralizing antibody in disposable plastic panels. Bull. Wld. Hlth. Org. 14:129-146. 1955.

Morgan, J. F., H. J. Morton and R. C. Parker
Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. 73:1-8. 1950.

Perreira, H. G., and A. E. H. Gampels
The growth of fowl plague and NDV in roller-tube cultures. J. Pathol. and Bacteriol. 67:109-115. 1954.

Plotz, H.
Etude comparative de la virulence des cultures de peste aviaire effectness en presence non proliferantes et en presence de cellues en voie de proliferation. Soc. biol. Compt. rend. 125:603-611. 1937.

Plotz, H., and B. Ephrussi
La cultures de la peste aviaire en presence de cellules vivantes non proliferantes. Soc. biol. Compt. rend. 113:711-712. 1933.

Reed, L. J., and H. Muench
A simple method of estimating fifty per cent endpoints. Am. J. hyg. 27:493-497. 1938.

Rightsel, W. A., P. Schultz, D. Muething and I. W. McLean
Use of vinyl plastic containers in tissue cultures for virus assays. J. Immunol. 76:464-475. 1956.

Rivers, T. M., E. Haagen and R. S. Muckenfuss
Development in tissue culture of the intracellular changes characteristic of vaccinal and herpetic infections. J. Exptl. Med. 50:665-672. 1929.

Rivers, T. M., and S. M. Ward
Jennerian prophylaxis by means of intradermal injections of culture vaccine virus. J. Exptl. Med. 62:549-560. 1935.

Robbins, F. C., and J. F. Enders
Tissue culture techniques in the study of animal viruses. Am. J. Med. Sci. 220:316-328. 1950.

Robbins, F. C., J. F. Enders and T. H. Weller
Cytopathogenic effect of poliomyelitis virus in vitro on human embryonic tissues. Proc. Soc. Exptl. Biol. Med. 75:370-374. 1952.

Rous, P., and F. S. Jones
A method for obtaining suspensions of living cells from the fixed tissues, and from plating out individual cells. Proc. Soc. Exptl. Biol. Med. 13:73. 1915.

Rubin, H., and R. M. Franklin
On the mechanism of Newcastle disease virus neutralization by immune serum. Virology. 3:84-95. 1957.

Salk, J. E., J. S. Younger and E. N. Ward
Use of color change of phenol red as an indicator in titrating poliomyelitis virus or its antibody in a tissue culture system. Am. J. Hyg. 60:214-230. 1954.

Smith, W. M., V. C. Chambers and C. A. Evans
Growth of neurotropic viruses in extraneural tissues: preliminary report on propagation of poliomyelitis virus: (Lansing and Hof. strains) in cultures of human testicular tissues. Northwest Med. 49:368-372. 1950.

Steinhardt, E., C. Israeli and R. H. Lambert
Studies on the cultivation of the virus of vaccinia. J. Infect Dis. 13:294-300. 1913.

Tapacio, T.
Pathology of the virus infected cell. Phillipines J. Sci. 53:245. 1934. Cited from Bang, F. B. Federation Proc. 14:619-624. 1955.

Theiler, M., and H. H. Smith
The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. J. Expt. Med. 65:767-786. 1937.

Tyrrell, D. A. J.
New tissue culture systems for influenza, Newcastle disease and vaccinia viruses. J. Immunol. 74:293-305. 1955.

Tyrrell, D. A. J., and F. L. Horsfall
Neutralization of viruses by homologous immune serum. I. Quantitative studies on factors which affect the neutralization reaction with Newcastle disease, influenza A and bacterial virus, T_3. J. Exptl. Med. 97:845-861. 1953.

Younger, J. S.
Demonstration of neutralizing antibodies for poliomyelitis viruses. Annals of the New York Academy of Sciences. 61:774-780. 1955.

Younger, J. S., E. N. Ward and J. E. Salk
Studies on poliomyelitis virus in cultures of monkey testicular tissue. II. Differences among strains in tissue culture infectivity with preliminary data on the quantitative estimation of virus and antibody. Am. J. Hyg. 55:301. 1952.

GROWTH AND TITRATION OF NEWCASTLE DISEASE AND INFECTIOUS BRONCHITIS VIRUSES IN TISSUE CULTURE

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Viruses grow only inside of cells. Because of this, virus research has been confined until recently to work with animals, either in the embryonic or adult stage. This presents many difficulties in attempting to perform quantitative work, the most obvious of which is variability among host animals. The introduction of tissue culture techniques presented methods whereby viruses could be handled in a manner simulating bacterial methodology.

Tissue culture techniques have permitted rapid advancement of virology, a spectacular step of which has been the development of the Salk vaccine and the quantitative titration of polio virus and antisera. Many of the techniques employed in the studies with poliomyelitis viruses have not as yet been extended to work with the other viruses. The purpose of this investigation was to develop tests similar to those used in poliomyelitis work for the titration of Newcastle disease and infectious bronchitis viruses.

Cell suspensions of chicken embryo tissues were obtained by trypsinization of chicken embryos at either 37 C or at 4 C. Cold trypsinization was found to give a high yield of viable cells even after 48 hours of trypsinization. Cell aggregation appeared to be less frequent when cold trypsinization was employed. HeLa cells were maintained and cell suspensions prepared by trypsinization at 37 C for 30 minutes. A hemocytometer was used to make direct counts of the number of cells in suspension. Minced allantoic and amniotic membranes were prepared by cutting them into small fragments of about 3 mm square.

The titration procedure that was developed utilized replicate tissue cultures, obtained by suspending the cells at the desired

concentration uniformly throughout the medium with a magnetic stirrer followed by delivery to culture vessels with a Cornwall automatic pipette.

Growth of Newcastle disease virus was demonstrated to make place in chick embryo fibroblasts and HeLa cells. Multiplication was recorded by EI_{50} titration in embryonated eggs and by observation of the cytopathogenic effects on Newcastle disease virus on the tissue culture cells.

It was not possible to show infectious bronchitis virus multiplication in chick embryo fibroblasts, chorioallantoic membranes or amniotic membranes. HeLa cells also did not support infectious bronchitis multiplication.

Infectious bronchitis virus was found to be heat labile. A preliminary experiment demonstrated quick freezing and subsequent storage at -60 C in a dry ice cabinet was a satisfactory method of preservation.

A method of titration of Newcastle disease virus which depends upon the color difference between infected and non-infected media in culture vessels was employed. The color difference was noted by the effect of pH of the fluid phase of the cultures as a result of the added phenol red and methylene blue. The titration procedure using color change in replicate tissue cultures was found sensitive enough to be used for neutralization tests employing Newcastle disease virus and Newcastle disease antiserum.

Although multiplication of infectious bronchitis virus in tissue culture could not be demonstrated, evidence was presented that

indicated infectious bronchitis virus had some effect on cell metabolism. This was seen by the difference in replicate tissue cultures inoculated with varying dilutions of infectious bronchitis virus.